FEBS Letters 580 (2006) 3321–3328

Nobiletin, a citrus flavonoid, suppresses phorbol ester-induced expression of multiple scavenger receptor genes in THP-1 human monocytic cells

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Received 18 February 2006; revised 14 April 2006; accepted 27 April 2006

Available online 4 May 2006

Edited by Laszlo Nagy

Abstract **Unregulated uptake of oxidized low-density lipoproteins (ox-LDL) via macrophage scavenger receptors (SRs) such as lectin-like ox-LDL receptor-1 (LOX-1) is a key event in atherosclerosis. In this study, we examined the effects of five selected food phytochemicals on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced LOX-1 mRNA expression in THP-1 human monocyte-like cells. Nobiletin, a citrus polymethoxylated flavone, markedly reduced it in dose- and time-dependent manners. It also suppressed the phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK) 1/2, and c-Jun (Ser-63), thereby inhibiting the transcriptional activity of activator protein-1. Further nobiletin attenuated expression of SR-A, SR-PSOX, CD36, and CD68, but not CLA-1, mRNA, leading to the blockade of DiI-acLDL uptake. Together, our results suggest that nobiletin is a promising phytochemical for regulating atherosclerosis with reasonable action mechanisms.**

Keywords: Oxidized LDL; Scavenger receptors; LOX-1; Nobiletin

1. Introduction

Atherosclerosis is now understood as a disease characterized by inflammation that results in a host of complications, including ischemia, acute coronary syndromes (unstable angina pectoris and myocardial infarction), and stroke. There is also consistent evidence that oxidized low-density lipoprotein (ox-LDL) is a key factor in the initiation and progression of the pathology of the disease [1–3]. Ox-LDL has diverse biological effects, including the expression of pro-inflammatory cytokines and adhesion molecules in arterial wall cells for accelerating atherogenesis [4], while it is also a chemoattractant for monocytes [5] and cytotoxic toward endothelial cells in culture [6]. After being recruited to subendothelial sites, monocytes differentiate into macrophages and express several distinct scavenger receptors (SRs), which mediate the endocytic uptake of ox-LDL. Further, macrophages are excessively and irreversibly loaded with ox-LDL, and then converted into foam cells because of the lack of a feedback inhibition mechanism for ox-LDL incorporation [1,7,8].

Thus far, SR-A (class A SR), CD36 and LIMPII analogous-1 (CLA-1); human orthologue of SR-BI and CD36 (class B SR), CD68 (class D SR), lectin-like oxidized LDL receptor-1 (LOX-1, class E SR), and SR for phosphatidylserine and ox-LDL (SR-PSOX or CXCL16, class G SR) have been shown to be responsible for cellular ox-LDL uptake [9]. It is of importance to note that ox-LDL increases the expression of those SRs, resulting in the enhancement of foam cell formation. The expression of SRs is induced by tumor necrosis factor-α (TNF-α), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and other factors [10,11], which suggests that the in vivo expression of SRs may be dynamically regulated by inflammatory and fluid mechanical stimuli. The importance of expression or repression of SRs in atherosclerosis has been demonstrated using transgenic and knockout mice. For example, SR-A knockout mice showed a significant decrease in atherosclerotic lesions as compared to apolipoprotein E knockout ($apoE^{-/-}$) [12] and LDL receptor knockout ($LDLR^{-/-}$) mouse models [13]. In addition, Febbraio et al. crossed $CD36^{-/-}$ mice with $apoE^{-/-}$ mice and reported a 70% reduction in atherosclerotic lesion size in animals fed a high-fat diet [14]. These findings suggest that the deletion or repression of SRs may lead to a predictable decrease in ox-LDL uptake and a resulting decrease in pathophysiological lesion formation, though a contradictory finding was recently presented [15].

Interestingly, the expressions of SRs were decreased by natural and synthetic peroxisome proliferator-activated receptor-gamma (PPARγ) ligands [16]. In addition, statins, which inhibit the key enzyme in cholesterol biosynthesis, have also been reported to downregulate those SRs [17], while aspirin inhibited ox-LDL-mediated LOX-1 expression [18]. Further, macrophages from mice given fish oil had a low level of expression of SR-A [19], while α-tocopherol

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Abbreviations: ACA, 1′-acetoxychavicol acetate; CLA-1, CD36 and LIMPII analogous-1; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; DMSO, dimethyl sulfoxide; EGCG, (−)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; IL, interleukin; LDL, low-density lipoprotein; LOX-1, lectin-like ox-LDL receptor-1; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; ox-LDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor-gamma; RT-PCR, reverse transcription-polymerase chain reaction; SR, scavenger receptor; SR-PSOX, SR for phosphatidylserine and ox-LDL; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.077

decreased the expression of CD36 in smooth muscle cells [20] and monocyte-derived macrophages [21]. Along a similar line, Teupser et al. showed that α- and β-tocopherol downregulate SR-A activity in rabbit peritoneal macrophages [22].

In the present study, we investigated the suppressive effects of five selected food phytochemicals on TPA-induced LOX-1 expression in a human acute monocytic leukemia cell line, THP-1, to extend our knowledge of agents that have physiological potentials for the regulation of SR expression. In our report, we also address the underlying molecular mechanisms of those effects.

2. Materials and methods

2.1. Materials

Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Gibco BRL (Grand Island, NY) and fetal bovine serum (FBS) came from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by Proligo (Kyoto, Japan). A QIAshredder™ and RNeasy Mini Kit® were purchased from Qiagen (Hilden, Germany), and an RNA PCR Kit (ver. 2.1, AMV) from TaKaRa Bio (Shiga, Japan). pRL-TK (containing the *Renilla* luciferase gene) and a Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Lipofectamine was purchased from Invitrogen. A Nuclear/Cytosol Fractionation Kit® was purchased from BioVision (Mountain View, CA). Antibodies were purchased from the following sources: rabbit anti-phospho-p38 mitogen-activated protein kinase (MAPK), rabbit anti-p38 MAPK, rabbit anti-phospho-JNK1/2, rabbit-anti-JNK1/2, rabbit anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-c-Jun (Ser-63), rabbit anti-phospho-c-Jun (Ser-73), rabbit anti-c-Jun, and rabbit antibody horseradish peroxidase-linked IgG antibodies came from Cell Signaling Technology (Beverly, MA); mouse anti-c-Fos and goat anti-β-actin antibodies came from Santa Cruz Biotechnology (Santa Cruz, CA); and horseradish peroxidase-conjugated anti-mouse IgG, and anti-goat IgG came from Dako (Glostrup, Denmark). PD98059, SB203580, and SP600125 were from Calbiochem (La Jolla, CA). Acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine (DiI) was obtained from Biomedical Technologies, Inc. (Stoughton, MA). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) unless specified otherwise.

2.2. Cell culture

Human monocyte-derived THP-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 ng/mL of streptomycin, 100 U/mL of penicillin, and 300 ng/mL of L-glutamine. The cells were incubated under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Cell suspensions were prepared at a concentration of 1.5×10^6 cells/1 mL (RPMI medium with FBS 10%), then incubated in 12-well plates for 30 min with each test sample at the specified concentrations, or with the vehicle (0.5% dimethyl sulfoxide; DMSO, v/v), followed by stimulation with TPA (30 nM) for the designated periods of time. The concentration of each agent used in the bioassays was set to be non-lethal maximum one, which was determined by preliminary experiments (data not shown). Total RNA was extracted using a QIAshredder™, RNase-free DNase sets, and an RNeasy Mini Kit®. A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) transcript served as the internal control. cDNA was synthesized using 1 μg of total RNA and an RNA PCR Kit (AMV). PCR amplification was performed using a thermal cycler (PTC-100™, MJ Research, Watertown, MA) and conducted with 0.05 μM of each sense and antisense primer. Primer sequences and PCR conditions (denaturation, annealing, and primer extension time and temperature) are listed in Table 1. PCR products were subjected to electrophoresis through 3% agarose gels and stained with SYBR® Gold. The signal intensities of the bands with expected sizes were quantified by NIH Image and compared with that of the internal standard GAPDH amplified under identical conditions.

2.4. Reporter assay

Cells were transfected with 4 μg of AP-1 promoter-luciferase constructs with the herpes simplex thymidine kinase driven *Renilla* luciferase reporter (pRL-TK) plasmid (Promega) using lipofectamine. Cells were incubated at a concentration of 2×10^6 cells/mL in Opti-MEM I medium containing the transfection mixture for 12 h at 37 °C. After transfection, the transfection reagent was replaced with RPMI medium containing 10% FBS for 12 h. Cells were plated in 24-well plates and treated with nobiletin (0–100 μM) for 30 min then stimulated with TPA (30 nM) for 48 h. pRL-TK was used in the co-transfection experiments to compare the transfection efficiencies. Firefly and *Renilla* luciferase were measured using the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's protocol. Briefly, cells were washed with phosphate-buffered saline (PBS) and lysed with passive lysis buffer (100 μl/well). The assays for firefly luciferase and *Renilla* luciferase activities were performed sequentially in a single reaction tube using a 20 μl aliquot of each cell lysate. The cell lysates were mixed with luciferase assay reagent II and firefly luminescence was measured using a luminometer with dual automatic injector (Lumat LB9507) (Perkin–Elmer, Boston, MA). Next, the samples were mixed with Stop and Glo reagent and *Renilla* luciferase activity was determined as an internal control. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

2.5. Western blotting

Cells (5×10^6 cells/3 mL RPMI medium with 10% FBS in a 60-mm dish) were incubated for 30 min with nobiletin (100 μM) or the vehicle (0.5% DMSO, v/v), then stimulated with TPA (30 nM) and collected after 0, 2 and 6 h. The concentration of each agent was determined based on previous cytotoxic experiments (data not shown). Cells were fractionated using a Nuclear/Cytosol Fractionation Kit®, with protease and phosphatase inhibitor cocktails (TaKaRa Bio), according to the recommendations of the supplier. The protein concentration in the cytosol fraction was determined using a DC protein assay (Bio-Rad Laboratories, Kyoto, Japan), with γ-globulin used as the standard. Denatured proteins (80 μg) were separated using SDS–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and transferred onto Immobilon-P Transfer Membranes (Millipore, Bedford, MA). After blocking overnight at 4 °C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were first incubated with each antibody at a dilution of 1:1000 [goat anti-β-actin, rabbit anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38 MAPK, rabbit anti-p38 MAPK, rabbit anti-phospho-JNK1/2, rabbit anti-JNK1/2, rabbit anti-phospho-c-Jun (Ser-73), rabbit anti-phospho-c-Jun (Ser-63), rabbit anti-c-Jun, and mouse anti-c-Fos (1:500 dilution)]. The second incubation was performed with horseradish peroxidase-conjugated secondary anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (1:1000 dilution each). The blots were developed using ECL Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and the intensity of each band was analyzed using NIH Image. Relative levels of each protein were corrected by employing β-actin as the internal standard.

2.6. Cellular uptake of DiI-acLDL

THP-1 cells were cultured on chamber slides (1×10^5 cells/0.2 mL RPMI medium with FBS 10%) and incubated for 30 min with the Nobiletin (100 μM) or the vehicle (0.5% DMSO, v/v). After being stimulated with TPA (30 nM), the cells were incubated at 37 °C for 24 h. After removal of the media, the cells were mounted in RPMI medium containing DiI-acLDL (10 μg/mL) and incubated at 37 °C for 4 h. The media was removed and the cells were washed three times with PBS, then fixed with 4% paraformaldehyde for 20 min at room temperature, followed by washing three times with distilled water at room temperature. Coverslips were inverted over a drop of 80% PBS in glycerol prior to viewing. DiI-acLDL uptake was observed with a fluorescence microscope UFX-35 A (Nikon, Tokyo).

Table 1
List of primer sequences for RT-PCR

Gene	Primer	Sequence (5′–3′)	Product size (bp)
LOX-1[a]	Forward	ACTCTCCATggTggTgCCTgg	251
	Reverse	CATTCAgCTTCCgAgCAAggg	
SR-A[b]	Forward	gCAgTTCTCATCCCTCTCATTggA	335
	Reverse	ATTCCCATgTCCCTgTggACTgAg	
CD36[c]	Forward	gAgACCTgCTTATCCAgAAgAC	510
	Reverse	gACCAACTgTggTAgTAACAgg	
CLA-1[d]	Forward	TgATgATggAgAATAAgCCCAT	696
	Reverse	TgACCgggTggATgTCCAggAAC	
CD68[e]	Forward	gCCACTCACAgTCCTgCCACC	426
	Reverse	ggACACATTgTACTCCACCgCC	
SR-PSOX[f]	Forward	TACACgAggTTCCAgCTCCT	154
	Reverse	gggggCTggTAggAAgTAAA	
CD11b[g]	Forward	TCggCggATgAAggAgTTTg	756
	Reverse	CTTTgCACCCggTTCCgTAAg	
CD18[h]	Forward	TCgTggACAAgACCgTgCTgC	421
	Reverse	CTACTggTCACCgCgAAgATCg	
GAPDH[i]	Forward	gCACCACCAACTgCTTAgCAC	636
	Reverse	gTCTgAgTgTggCAgggACTC	

[a]40 s denaturation at 96 °C, 30 s of annealing at 60 °C, and 90 s of primer extension at 72 °C.
[b–d]40 s at 96 °C, 30 s at 55 °C, and 90 s at 72 °C.
[e]40 s at 96 °C, 30 s at 62 °C, and 90 s at 72 °C.
[f,i]30 s at 95 °C, 60 s at 58 °C, and 60 s at 72 °C.
[g,h]60 s at 96 °C, 60 s at 56 °C, and 120 s at 72 °C.
[a–f]32.
[g]28.
[h]21.
[i]20 cycles.

2.7. Statistical analysis

Each experiment was performed at least three times and data are shown as the means ± standard deviation (S.D.) where applicable. Statistically significant differences between groups in each assay were determined using a Student's *t*-test (two-sided).

3. Results

3.1. Effects of selected food phytochemicals on TPA-induced LOX-1 mRNA expression in THP-1 cells

The food phytochemicals used in the present study were selected based on their pronounced anti-inflammatory properties shown in in vitro and in vivo studies reported previously [23–26]. In order to examine their effects on *LOX-1* mRNA expression, THP-1 human monocytic cells were pre-treated with each of the five tested food phytochemicals [1′-acetoxychavicol acetate (ACA) at 20 μM, auraptene at 100 μM, nobiletin at 100 μM, (−)-epigallocatechin-3-gallate (EGCG) at 100 μM, resveratrol at 20 μM] or the vehicle, followed by TPA exposure for 24 h. As shown in Fig. 1A, the vehicle-treated THP-1 cells scarcely expressed *LOX-1* mRNA, as detected by RT-PCR, whereas treatment with TPA highly induced it by 7.3-fold, while nobiletin attenuated it by 75%. In contrast, ACA, auraptene, EGCG, and resveratrol increased the expression by 1.7- to 3.0-fold.

3.2. Concentration- and time-dependency of nobiletin on LOX-1 mRNA expression suppression

As shown in Fig. 1B, TPA-treated THP-1 cells increased LOX-1 mRNA levels in a time-dependent fashion from 6 to 24 h. Pretreatment with nobiletin (0.8–100 μM) for 24 h markedly decreased TPA-induced *LOX-1* mRNA in a concentration-dependent manner, with the 50% inhibition concentration found to be 2.9 μM.

3.3. Nobiletin inhibited AP-1 transactivation

The expression of *LOX-1* is regulated by several transcriptional factors, including AP-1 [27]. To evaluate the mechanism by which nobiletin downregulates the expression of *LOX-1*, we examined its inhibitory effects on TPA-induced activation of AP-1. TPA-treatment led to a sevenfold increase in AP-1 transcription activity (Fig. 1C), while a concentration-dependent suppression ranging from 35% to 66% was seen when the cells were pre-treated with nobiletin in a concentration range of 4–100 μM.

3.4. Nobiletin inhibited MAPK activation

Activation of the MAPK cascade is well known to contribute to AP-1 transactivation. Therefore, we pre-treated THP-1 cells with nobiletin (100 μM) or the vehicle for 30 min and then stimulated them with TPA for 0, 2, and 6 h. The cytosolic pro-

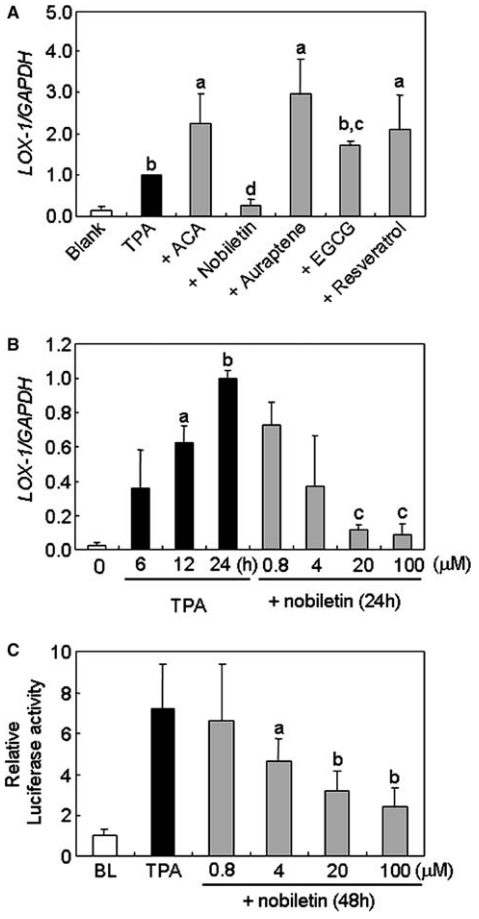

Fig. 1. (A) Effects of food phytochemicals on TPA-induced *LOX-1* mRNA expression in THP-1 cells. Cells (1.5×10^6 cells/mL) were incubated separately for 30 min with each of five food phytochemicals [ACA (20 μM), nobiletin (100 μM), auraptene (100 μM), EGCG (100 μM), and resveratrol (20 μM)] or the vehicle, followed by stimulation with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR as described in Section 2. *LOX-1* mRNA levels were normalized to the levels of GAPDH mRNA. $^{a}P < 0.005$, $^{b}P < 0.05$ versus blank. $^{c}P < 0.005$, $^{d}P < 0.01$ versus TPA. Data are expressed as the means ± S.D. of three independent experiments. (B) Dose-dependent effect of nobiletin on TPA induced LOX-1 mRNA expression in THP-1 cells. Cells (1.5×10^6 cells/mL) were cultured with the vehicle for 30 min followed by stimulation with TPA (30 nM) for 6, 12, or 24 h then cultured with nobiletin at a concentration of 0.8, 4, 20, or 100 μM for 30 min followed by stimulation with TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR. *LOX-1* mRNA levels were normalized to the levels of GAPDH mRNA. Data are expressed as the means ± S.D. of three experiments. $^{a}P < 0.01$, $^{b}P < 0.001$ versus blank. $^{c}P < 0.001$ versus TPA. (C) THP-1 cells were transfected with AP-1 promoter-luciferase constructs with the pRL-TK plasmid using lipofectamine for 12 h at 37 °C. Next, the transfection reagent was replaced by RPMI medium containing 10% FBS and the cells were incubated for an additional 12 h. The cells were plated in 24-well plates and treated with nobiletin (0.8–100 μM) or the vehicle for 30 min then stimulated with TPA (30 nM) for 48 h. Firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's protocol. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to that of *Renilla* luciferase. $^{*}P < 0.005$, $^{**}P < 0.001$ versus TPA.

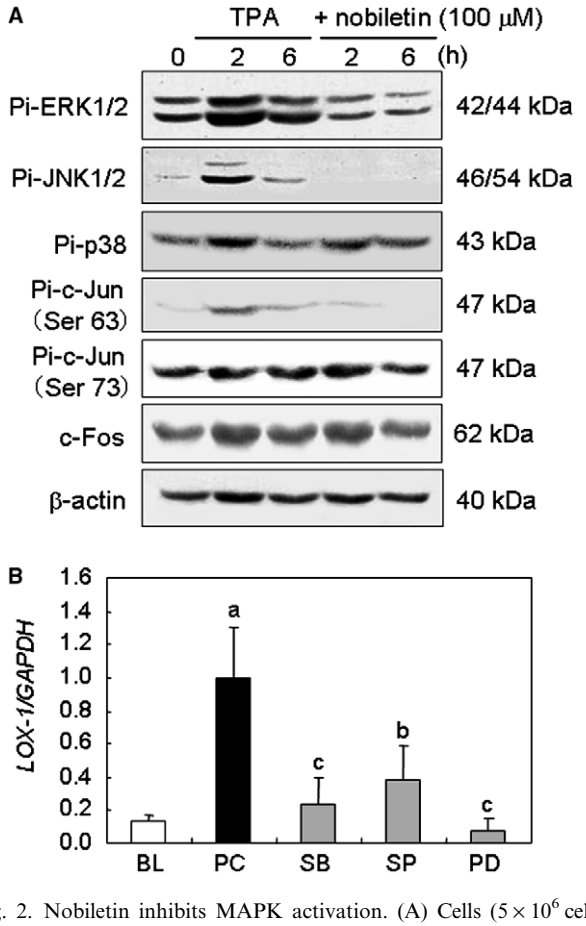

Fig. 2. Nobiletin inhibits MAPK activation. (A) Cells (5×10^6 cells/3 mL) were incubated for 30 min with nobiletin (100 μM) or the vehicle, then stimulated with TPA (30 nM) and collected after 0, 2, and 6 h. The cells were fractionated into cytosolic fractions as described in Section 2. Each protein in the cytosolic fraction was subjected to Western blotting with antibodies for phospho-ERK1/2, phospho-JNK1/2, phospho-p38, phospho-c-Jun (Ser-63), phospho-c-Jun (Ser-73), c-Fos, and β-actin. Three independent experiments were performed, with representative results shown. (B) Pharmacological effects toward TPA-induced *LOX-1* gene expression. THP-1 cells were pretreated separately with each of the specific inhibitors [SB, SB203580 (50 μM); SP, SP600125 (20 μM); PD, PD98059 (100 μM)] for 30 min before exposure to TPA (30 nM) for 24 h. At the end of the incubation period, the cells were lysed and *LOX-1* mRNA was analyzed by RT-PCR. *LOX-1* mRNA levels were normalized to the levels of GAPDH mRNA. Data are expressed as the means ± S.D. of three experiments. $^{a}P < 0.05$ versus blank, $^{b}P < 0.005$ versus TPA.

teins thus obtained were subjected to Western blot analysis to detect both inactive and activated forms of p38 MAPK, JNK1/2, and ERK1/2, using specific antibodies. As shown in Fig. 2A, following TPA treatment, those kinases were markedly and transiently phosphorylated after 2 h, whereas nobiletin blocked the TPA-enhanced activation of ERK1/2 and JNK1/2, but not that of p38 MAPK. In addition, the flavonoid significantly reduced c-Jun (Ser-63, but not Ser-73) phosphorylation, while c-Fos expression was not affected. The expression levels of the inactive forms of MAPKs were not significantly changed (data not shown).

3.5. Pharmacological effects on TPA-induced LOX-1 gene expression

To identify the signaling pathways involved in TPA-induced *LOX-1* mRNA expression, THP-1 cells were pre-treated with each of the specific inhibitors for 30 min before exposure to TPA for 12 h. As shown in Fig. 2B, pretreatment with

SB203580 (p38 MAPK inhibitor, 50 μM), SP600125 (JNK1/2 inhibitor, 20 μM), or PD98059 (MEK1/2 inhibitor, 100 μM) significantly suppressed TPA-induced *LOX-1* mRNA expression in a range of 62–92%.

3.6. Effects toward other SRs and adhesion molecules expression

Next, we examined the effects of nobiletin on the mRNA expression of other SRs (*SR-A*, *CD36*, *CD68*, *SR-PSOX*, and *CLA-1*) as well as adhesion molecules (*CD11b* and *CD18*) using RT-PCR. THP-1 cells were pre-treated with nobiletin at 100 μM or the vehicle, followed by TPA exposure for 24 h. As shown in Figs. 3A and B, cells not treated with TPA expressed scant levels of *SR-A*, *SR-PSOX*, *CD11b*, and *CD18* mRNA, while those were significantly upregulated with TPA treatment. In addition, *CD36*, *CD68*, and *CLA-1* mRNA was highly expressed in a constitutive manner in non-treated THP-1 cells and *CD68* mRNA expression was upregulated twofold after TPA exposure. Nobiletin abolished the mRNA expression of *SR-A*, *SR-PSOX*, *CD36*, *CD11b*, and *CD18*, while that of *CD68* was decreased to the basal level.

3.7. Nobiletin inhibited DiI-acLDL uptake

The above results led us to examine the effect of nobiletin on cellular uptake of modified LDL. DiI-acLDL is known to be bound to and/or taken up via type A scavenger receptors. THP-1 cells were pre-treated with 100 μM of nobiletin or the vehicle for 30 min and then stimulated with TPA for 24 h. Next, the cells were exposed to DiI-acLDL for 4 h at 37 °C.

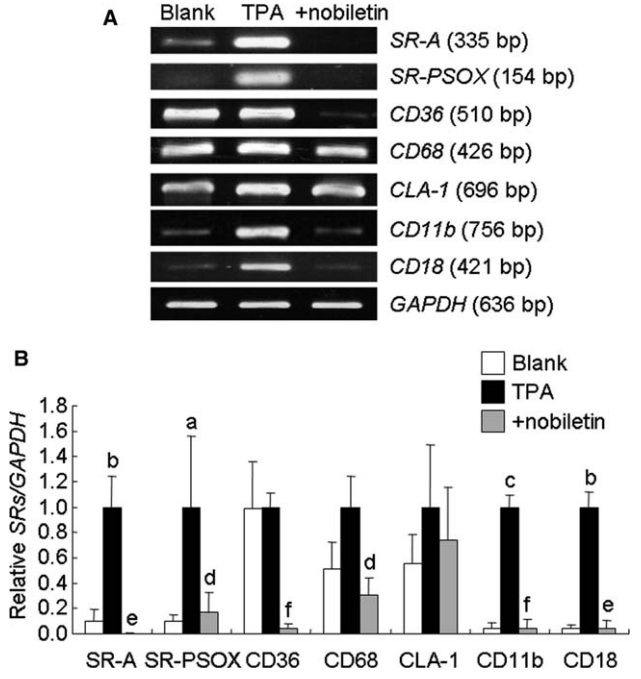

Fig. 3. Suppressive effects of nobiletin on the expression of several different SRs and adhesion molecules. The cells (1.5×10^6 cells/ml) were incubated with 100 μM of nobiletin or the vehicle for 30 min followed by stimulation with TPA (30 nM) for 24 h. mRNA from each sample was quantified by RT-PCR, using GAPDH as the standard, described in Section 2. (A) Three independent experiments were performed, with representative data shown. (B) Each bar represents the mean ± S.D. $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, $^{\#\#\#}P < 0.001$ versus blank. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ versus TPA.

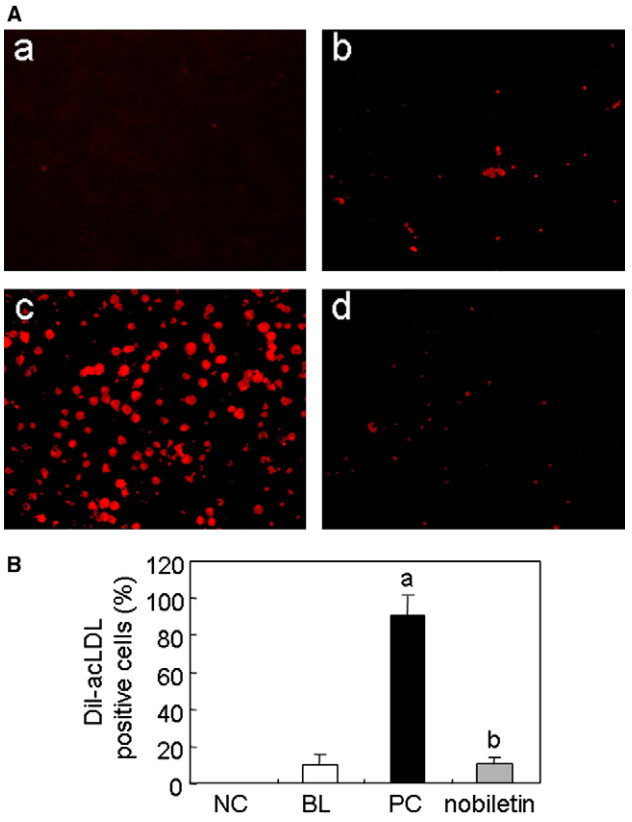

Fig. 4. Suppressive effects of nobiletin on uptake of DiI-acLDL. (A) THP-1 cells (2.5×10^5 cells/ml) were incubated with nobiletin (100 μM) or the vehicle for 30 min and stimulated with TPA (30 nM) for 24 h at 37 °C. Then, the cells were washed three times with PBS and incubated with DiI-acLDL (10 μg/ml) for 4 h at 37 °C. As a negative control, cells were incubated without DiI-acLDL. DiI-acLDL uptake was assessed by fluorescence photomicroscopy. The experiments were repeated three times independently, with one representative result for each shown. (a) Negative control, (b) blank, (c) TPA, (d) nobiletin + TPA. Approximately 100 cells are shown in each photograph. (B) Data are expressed as the means ± S.D. ($n = 3$). $^{a}P < 0.005$ versus blank. $^{b}P < 0.005$ versus TPA.

No DiI-acLDL uptake by undifferentiated THP-1 cells was observed, whereas TPA treatment led to an increased uptake (Fig. 4A), which was abolished by nobiletin.

4. Discussion

Macrophages have a central role as effector cells at sites of chronic inflammation, such as those associated with atherosclerotic plaque. The expression of SRs is upregulated during the differentiation of monocytes into macrophages, which is a key event in the process of atherosclerosis. SRs excessively uptake ox-LDL through SRs, leading to their conversion into foam cells [1,7,8]. They are known to be regulated by TNF-α, TPA, and ox-LDL [10,11], suggesting that SRs expression in vivo may be dynamically regulated by inflammatory and fluid mechanical stimuli. Several studies have also shown that targeted deletion of either SR-A or CD36 in hyperlipidemic mouse models leads to a reduction in atherosclerotic lesions [12–14]. Similarly, Kunjathoor et al. reported that macrophages lacking both SR-A and CD36

demonstrated a reduction of 80–90% in internalized and degraded ox-LDL and acLDL [28]. Those findings suggest that deletion or repression of SRs may predictably decrease ox-LDL uptake and thereby reduce pathophysiological lesion formation. However, there are only a few studies regarding the suppressive effects of food factors on SRs expression [19–22]. Our results showed that the five selected food phytochemicals had effects on TPA-induced SRs expression in THP-1 cells and elucidated some of the underlying molecular mechanisms.

Nobiletin, a polymethoxylated flavone found specifically in citrus fruits [29], is believed to be a promising anti-inflammatory and anti-tumor promoting agent [23,30–33]. We previously reported that nobiletin acts as a dual inhibitor of superoxide and nitric oxide generation in inflammatory leukocytes, and also showed its suppressive effects toward the formation of inflammatory mediators and tumor promotion in mouse skin [23,31]. Further, nobiletin has been found to suppress the induction of matrix metalloproteinase (MMP)-7 [32] and MMP-9, as well as the release of prostaglandin E_2 in rabbit synovial fibroblasts [33]. In addition, Lin et al. reported that nobiletin suppressed the expression of proinflammatory cytokines, including interleukin-1 (IL-1), IL-6, and TNF-α, in mouse macrophages [30]. Those cytokines are involved in various pathological conditions such as atherosclerosis and hypertension [34], thus nobiletin may demonstrate a suppressive ability at sites of chronic inflammation.

Other compelling evidence indicates that citrus flavonoids, including nobiletin, can suppress the hepatic production of cholesterol-containing lipoproteins, thus reducing the total cholesterol concentration in plasma-, and leading to a reduction in the occurrence of cardiovascular disease [35,36]. Nobiletin has been shown to have a hepatic apolipoprotein B-lowering potential in vitro [36,37], while recent results suggested that it can reduce the circulating concentrations of very low density lipoproteins and LDL in blood, and directly inhibit macrophage-derived foam cell formation at the site of lesion development within vessel walls [38,39]. Whitman et al. showed that nobiletin reduced the accumulation of cholesterol ester mediated by acLDL (a known ligand for SR-A) in the mouse macrophage line J774A [39]. Therefore, it is possible that nobiletin inhibits the process of acLDL internalization mediated by SR-A, though it did not show an effect toward total and surface SR-A protein expression levels [39]. The action mechanisms underlying the anti-atherogenic effects of nobiletin described above remain to be clarified.

In the present study, we showed for the first time that nobiletin suppresses the expression of multiple SR and adhesion molecule genes induced by phorbol ester, such as that of *LOX-1*, *SR-A, SR-PSOX*, *CD68*, *CD36, CD11b, and CD18*. The relevance of suppression of their expression by nobiletin in vitro in the process of preventing the initiation and progression of atherosclerosis in vivo remains unclear. Tsukamoto et al. analyzed the expressions of SRs at different stages of differentiation from THP-1 monocytes to foam cells, and their results suggested that CD36, CLA-1, and CD68, but not SR-A or LOX-1, play crucial roles in the progression to foam cells from macrophages [40]. We believe that our results also provide insight into the molecular mechanisms, whose understanding is necessary to explain the anti-atherogenic actions of this flavonoid.

Treatment of THP-1 cells with TPA induces a program of macrophage differentiation and marked upregulation of SR genes [41]. The 5′ flanking region of the human *LOX-1* gene contains multiple putative binding sites for several transcription factors, including those of AP-1, GATA family, STAT family, NF-IL6, Oct-1, CCAAT enhancer-binding proteins, and cyclic AMP response element binding protein [27]. Protein kinase C activated by TPA is known to stimulate the activities of several classes of transcription factors, including AP-1 [42]. The present results suggest that the inhibitory effects of nobiletin toward TPA-induced *LOX-1* expression are partly associated with the suppression of AP-1 activity (Fig. 1C). This notion is supported by several previous findings, while we recently found that nobiletin downregulates MMP-7 expression in HT-29 human colorectal cancer cells via a reduction in AP-1 DNA binding activity [32]. Along a similar line, Sato et al. demonstrated that nobiletin inhibits the invasive activity of TPA stimulated-human fibrosarcoma HT-1080 cells by suppressing AP-1 binding activity [30].

AP-1 transcriptional activity is dependent on both ERK1/2 and JNK1/2 MAPK-mediated signaling pathways for c-Fos and c-Jun induction, as well as c-Jun phosphorylation. In the present study, nobiletin inhibited the phosphorylation of JNK1/2 (Fig. 2A), which presumably results in decreased JNK1/2 activity, leading to a reduced level of c-Jun phosphorylation and blocking of AP-1 transcription activity. Previous reports have shown that ERK1/2 activation contributes to AP-1 activation by Elk-1 phosphorylation, which induces c-Fos synthesis. Although nobiletin inhibited the phosphorylation of ERK1/2, c-Fos protein levels did not change within 6 h. Thus, it remains unclear whether ERK1/2 repression by nobiletin plays a role in AP-1 activation.

Nobiletin is a hydrophobic molecule that has six methoxyl groups, which is a characteristic associated with a high cellular uptake rate in vitro as compared with general flavonoids [43]. In an in vivo study as well, we showed that nobiletin exhibits greater localization in the mucosa and muscularis in the gastrointestinal tract as compared to luteolin, a flavonoid with four hydroxyl groups [44]. Thus, it is important to examine the ability of nobiletin to absorb into and localize within blood vessel walls.

In conclusion, we found that nobiletin has a distinct ability to suppress the expression of multiple SRs induced by TPA via AP-1 repression, which blockaded DiI-acLDL uptake. Our results suggest that this flavonoid may be effective as an agent to regulate the development of atherosclerosis. Further studies utilizing atherosclerosis-prone animal models are needed to clarify our findings.

Acknowledgements: This research was supported by Grants-in-Aid from the Ministry of Agriculture, Forestry and Fisheries of Japan and from Japan Science and Technology Agency.

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